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Investigation of Genotypes of *Borrelia burgdorferi* in *Ixodes scapularis* Ticks Collected during Surveillance in Canada▽

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The genetic diversity of *Borrelia burgdorferi* sensu stricto, the agent of Lyme disease in North America, has consequences for the performance of serological diagnostic tests and disease severity. To investigate *B. burgdorferi* diversity in Canada, where Lyme disease is emerging, bacterial DNA in 309 infected adult *Ixodes scapularis* ticks collected in surveillance was characterized by multilocus sequence typing (MLST) and analysis of outer surface protein C gene (*ospC*) alleles. Six ticks carried *Borrelia miyamotoi*, and one tick carried the novel species *Borrelia kurtenbachii*. 142 ticks carried *B. burgdorferi* sequence types (STs) previously described from the United States. Fifty-eight ticks carried *B. burgdorferi* of 1 of 19 novel or undescribed STs, which were single-, double-, or triple-locus variants of STs first described in the United States. Clonal complexes with founder STs from the United States were identified. Seventeen *ospC* alleles were identified in 309 *B. burgdorferi*-infected ticks. Positive and negative associations in the occurrence of different alleles in the same tick supported a hypothesis of multiple-niche polymorphism for *B. burgdorferi* in North America. Geographic analysis of STs and *ospC* alleles were consistent with south-to-north dispersion of infected ticks from U.S. sources on migratory birds. These observations suggest that the genetic diversity of *B. burgdorferi* in eastern and central Canada corresponds to that in the United States, but there was evidence for founder events skewing the diversity in emerging tick populations. Further studies are needed to investigate the significance of these observations for the performance of diagnostic tests and clinical presentation of Lyme disease in Canada.

Lyme disease risk is currently emerging in eastern and central Canada due to northern expansion of the range of the tick vector *Ixodes scapularis* (30, 31). Studies in one part of the zone of emergence in Canada suggest that founder populations of the agent of Lyme disease, *Borrelia burgdorferi*, comprise genotypes introduced from the northeastern United States, most likely by migratory birds (29, 31). It has been hypothesized that the agent of Lyme disease in North America, *B. burgdorferi*, is currently undergoing a period of adaptive radiation (23). The resulting genetic diversity of the bacterium could have consequences for disease severity and clinical symptoms observed in infected animals and humans (18, 33, 36, 37, 38), as well as for the performance of some diagnostic tests (17, 39).

In this study, we have investigated diversity by two methods: housekeeping genes located on the main linear chromosome (by multilocus sequence typing [MLST] [24, 25]), which are under purifying selection and evolve nearly neutrally, and the plasmid-borne outer surface protein C (*ospC*) gene, which is under balancing selection, and consequently, analyses of MLST and *ospC* alleles are not correlated on large geographic scales (24). The balancing selection of *ospC* is thought to be driven by host immune responses, because it is expressed early during *B. burgdorferi* infection of vertebrate hosts (32, 35). However, some researchers have raised the hypothesis that multiple-niche polymorphism and fitness variation of *B. burgdorferi* carrying different *ospC* alleles in diverse reservoir host communities explains the observed evidence for balancing selection (4). This may mean that geographic variations in host community assemblages drive geographic variation in the frequency of *ospC* alleles.

We have investigated two hypotheses. The first is that *B. burgdorferi* in ticks collected in Canada in passive surveillance are being dispersed from the United States into Canada by migratory birds and thus will carry the same MLST types that are found in the United States, with geographic patterns similar to those seen in the United States (15). Second, *ospC* may be a more direct indicator of clinically and diagnostically significant diversity of *B. burgdorferi* than MLST. If ticks carrying *B. burgdorferi* in passive surveillance in Canada are mostly dispersed from the United States into Canada by migratory birds, *ospC* diversity should also be the same in Canada as in the United States (29).

These investigations have practical importance in helping us to understand the pattern of emergence of tick and *B. burgdorferi* populations in Canada, whether patterns in Canada are different from those in the United States, and whether the

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ongoing range expansion of the tick vector may have implications for the bacterial populations with an effect on clinical symptoms and diagnosis in affected patients.

MATERIALS AND METHODS

Samples used in this study. *I. scapularis* ticks were collected from companion animals and humans by veterinary clinics and medical clinics from Alberta to Newfoundland in Canada between 2005 and 2007 as part of the national passive surveillance system (27, 31). The ticks were tested at the National Microbiology Laboratory of the Public Health Agency of Canada for *B. burgdorferi* infection. DNA was purified using a Qiagen DNeasy 96 Tissue kit (Qiagen Inc., Mississauga, ON, Canada) optimized for recovery of low-copy-number DNA from ticks. Real-time PCR targeting the 23S rRNA locus and *ospA* was used to screen the ticks for *Borrelia* infection, as described previously (7, 27). Only data from ticks that had tested positive by this method were used in the genetic and statistical analyses described below.

***ospC* analysis.** The *ospC* alleles carried by *B. burgdorferi* in infected ticks were identified by reverse line blot (RLB) hybridization as previously described (29). Briefly, a 522-bp region of the *ospC* gene of *B. burgdorferi* was amplified by a seminested PCR using the external primers OC6 (+) and OC623 (−) and internal primers OC6 (+Fluo) and OC602 (−), which target conserved regions, as previously described (4). The amplicons were probed with *ospC* type-specific probes by RLB as previously described (4, 32).

In some samples, identified by RLB as carrying single-allele (i.e., unmixed) infections, *ospC* was amplified by nested PCR using primers and conditions described by Bunikis and coauthors (6). Amplicons were sequenced in both directions in order to identify the *ospC* allele carried by *B. burgdorferi* in that tick. The sequences were then aligned and compared with reference sequences downloaded from GenBank using the ClustalW algorithm implemented in MEGA version 3.1 (20). The reference sequences were as follows: type A, AF029860; type B, AF029861; type C, AF029862; type D, AF029863; type E, AF029864; type F, AF029865; type G, AF029867; type H, AF029868; type I, AF029869; type J, AF029870; type K, AF029871; type L, L42899; type M, U01892; type N, L42897; type O, X84778; type T, AF065143; and type U, AF065144. The criterion for inclusion within an *ospC* type was ≥99% similarity, and the criterion for exclusion from an *ospC* type was ≤90% similarity (32).

MLST and phylogenetic analyses. Most tick samples shown by RLB to be infected with *B. burgdorferi* carrying single *ospC* alleles (suggesting that the ticks were less likely to carry *B. burgdorferi* of mixed STs) were used for MLST as described previously (24, 25). Briefly, eight housekeeping genes were amplified by nested PCR and sequenced by the Genomics Core Facility at the National Microbiology Laboratory of the Public Health Agency of Canada. Sequences of individual genes were compared with sequences present in the *Borrelia* MLST database at http://borrelia.mlst.net to obtain allele numbers and to determine the allelic profile and sequence types (STs) of samples. New alleles were compared with each other using the nonredundant database (NRDB) software available on the MLST website (http://www.mlst.net), and consecutive numbers were given to each distinct new allele. All sequence data for housekeeping genes are available at the MLST website hosted at Imperial College London, London, United Kingdom (http://borrelia.mlst.net). To ensure that *Borrelia* DNA in tick samples was capable of being amplified and to identify false-negative results, all tick samples subjected to MLST analysis were also tested by a *Borrelia*-specific PCR targeting the 16S-23S intergenic spacer (IGS) region (6).

Allelic profiles were analyzed using eBurst (9) and goeBurst (global optimal eBurst) (11). eBurst is based on a simple model of clonal expansion and divergence and provides a convenient method to establish relationships of descent for bacterial populations (http://www.mlst.net [9]). An implemented bootstrap procedure can give statistical confidence to the assignment of clonal complex founders. goeBurst, a further development of eBurst, has some additional features, such as a global optimization procedure (instead of local optimization) and an extended set of tie break rules, and allows better graphical representation of clonal complexes, including double-locus variants (DLV) and triple-locus variants (TLV) (11). Both algorithms are tailored for the use of MLST data and cluster STs as disjointed tree collections based on a set of hierarchical rules related to the number of single-locus variants (SLV), DLV (eBurst), and TLV (goeBurst). To obtain bootstrap support for the founder strains of clonal complexes for *B. burgdorferi*, we included all STs found in Canada and added 78 previously described STs from the northeastern (NE) and Midwestern (MW) United States (15) to an eBurst analysis. The minimum number of identical loci for group definition was set to 5, and the minimum count of SLV for subgroup definition was set to 0. The same samples were used in goeBurst to obtain a graphical display of clonal complexes.

For phylogenetic analyses, the sequences of all eight housekeeping genes were concatenated, and trees were generated using the maximum-likelihood method available on the PhyML website (http://www.atgc-montpellier.fr/phyml) (12). The settings were as follows: GTR was chosen as a substitution model, and the gamma parameter, invariant sites, and DNA models were estimated. For tree improvements, two methods, nearest-neighbor interchange (NNI) and subtree pruning and regrafting (SPR), were employed (16). Approximate likelihood ratios (aLRT) were calculated using the Shimodaira-Hasegawa (SH)-like procedure (2).

Statistical analysis. We investigated the occurrence of spatial clustering in identified MLST STs and *ospC* alleles using cluster analysis in SaTScan version 8.0 and a Bernoulli model (19) with a temporal precision of 1 year. The maximal spatial cluster size was set at 50% of the population, and the latitudes and longitudes for each submitted tick were those obtained from Natural Resources Canada (Geographical Names of Canada [http://gnss.nrcan.gc.ca/gnss-srt/searchName.jsp?language=en]) for the town or village of origin identified on the submission. A number of outcomes were investigated by this method. First the region of origin (MW or NE United States) of STs that were identical to STs already identified in studies in the United States (15, 24) was investigated to see if STs already identified in the United States have a similar geographic distribution in Canada. Second, the clustering of STs newly identified in this study or present in the MLST database but not yet further described was investigated to see if there was a geographic pattern to STs that to date are unique to Canada. Third, the spatial clustering of each *ospC* allele was investigated to provide initial investigation of possible geographic variations in *ospC* allele frequencies.

Multiple pairwise comparisons among *ospC* alleles were carried out using generalized linear models with logi link function in STATA/SE for Windows (Statacorp LP, TX) to investigate whether there were significant differences in the likelihoods that different alleles occurred together in mixed infections. In each case, multivariable models were created for each allele with all the other alleles considered explanatory variables. These models were reduced to the most parsimonious model by stepwise elimination of explanatory variables. Because these multiple comparisons are prone to type I errors, correspondence analysis was conducted on the data set in SAS version 9.2 (Cary, NC) to see if associations observed in pairwise comparisons were supported when accounting for the full variance-covariance matrix of the data. The level of significance was a P value of <0.05 throughout.

RESULTS

MLST typing. Of the tick samples suitable for MLST, 180 were randomly selected for analysis. Eighteen of them showed mixed infections after sequencing of one or more of the housekeeping genes, and PCR products were not obtained for one or more genes in a further 20 cases. Six of the latter samples, three from Nova Scotia and three from Ontario, produced 16S-23S IGS amplicons that yielded products of 500 to 600 bp rather than the 804 to 812 bp expected for *B. burgdorferi* (6). The sequenced products from two of these ticks were qualitatively sufficient to be compared to sequences available in GenBank and showed the highest similarity to *Borrelia miyamotoi* (GenBank accession number AY531879.1). Therefore, we suggest that these ticks carried relapsing-fever-like spirochetes, likely to be *B. miyamotoi* by PCR of the 16S-23S intergenic spacer region. Usable sequences of all eight (MLST) housekeeping genes of *B. burgdorferi* sensu lato were amplified from 136 samples, and 30 previously published samples from Quebec were included in the study (31). The 166 samples for which sequences were obtained originated from the Atlantic provinces (New Brunswick, Nova Scotia, Prince Edward Island, and Newfoundland; $n = 61$), Ontario ($n = 67$), Quebec ($n = 31$, including those analyzed in a previous study [31]), Manitoba ($n = 6$), and Alberta ($n = 1$) (Table 1).

A total of 146 ticks carried *B. burgdorferi* STs that had previously been described from the NE and MW United States

TABLE 1. Sequence types and allelic profiles of *B. burgdorferi* in ticks collected in surveillance in Canada from 2005 to 2007

NML ID no.[a]	P/T[b]	ST[c]	Allele no.							
			clpA	*clpX*	*nifS*	*pepX*	*pyrG*	*recG*	*rplB*	*uvr*
AB07-3	AB	**300**	8	1	1	14	2	16	1	10
MB07-20	MB	**301**	12	1	1	7	112	6	1	10
MB07-21	MB	12	3	3	2	4	3	4	4	4
MB07-22	MB	**302**	5	5	4	114	1	15	1	6
MB07-25	MB	29	18	12	1	11	2	15	1	2
UMB06-10	MB	12	3	3	2	4	3	4	4	4
UMB07-5	MB	**221**	3	3	2	2	3	4	4	4
NB06-101	NB	4	8	1	1	1	4	6	1	7
NB06-127	NB	14	9	1	1	7	1	6	1	10
NB06-129	NB	1	1	1	1	1	1	1	1	1
NB06-48	NB	7	6	1	5	1	1	7	1	8
NB06-5	NB	1	1	1	1	1	1	1	1	1
NB06-85	NB	18	7	6	6	1	1	5	5	5
NB07-119	NB	3	4	1	1	1	1	6	1	7
NB07-121	NB	3	4	1	1	1	1	6	1	7
NB07-137	NB	9	10	5	4	6	1	6	1	6
NB07-17	NB	4	8	1	1	1	4	6	1	7
NB07-50	NB	36	10	5	4	6	1	15	1	6
NB07-87	NB	**305**	5	5	4	5	5	5	94	6
NB07-95	NB	1	1	1	1	1	1	1	1	1
NF06-4	NFL	19	4	4	3	3	3	3	3	3
NS05-104	NS	**306**	5	5	4	5	5	6	1	6
NS06-10	NS	1	1	1	1	1	1	1	1	1
NS06-100	NS	9	10	5	4	6	1	6	1	6
NS06-113c	NS	18	7	6	6	1	1	5	5	5
NS06-129	NS	1	1	1	1	1	1	1	1	1
NS06-151	NS	7	6	1	5	1	1	7	1	8
NS06-196a	NS	36	10	5	4	6	1	15	1	6
NS06-2	NS	16	2	2	1	2	2	2	2	2
NS06-233	NS	7	6	1	5	1	1	7	1	8
NS06-234	NS	**307**	9	1	1	7	1	1	1	10
NS06-235	NS	1	1	1	1	1	1	1	1	1
NS06-240c	NS	1	1	1	1	1	1	1	1	1
NS07-121	NS	**281**	127	88	87	107	105	108	88	98
NS07-125	NS	59	6	1	5	1	1	7	1	19
NS07-128	NS	38	21	1	15	8	1	18	4	7
NS07-166	NS	3	4	1	1	1	1	6	1	7
NS07-279	NS	36	10	5	4	6	1	15	1	6
NS07-28	NS	**308**	4	91	1	1	1	6	1	7
NS07-290	NS	7	6	1	5	1	1	7	1	8
NS07-2a	NS	3	4	1	1	1	1	6	1	7
NS07-68	NS	9	10	5	4	6	1	6	1	6
NS07-75	NS	37	7	6	12	1	1	5	5	5
NS07-20	NS	**309**	7	6	12	115	1	114	5	5
NS07-96	NS	18	7	6	6	1	1	5	5	5
ON06-102	ON	9	10	5	4	6	1	6	1	6
ON06-107	ON	14	9	1	1	7	1	6	1	10
ON06-108	ON	38	21	1	15	8	1	18	4	7
ON06-187c	ON	19	4	4	3	3	3	3	3	3
ON06-188	ON	1	1	1	1	1	1	1	1	1
ON06-190	ON	3	4	1	1	1	1	6	1	7
ON06-194	ON	**226**	8	1	15	94	2	20	1	7
ON06-231	ON	1	1	1	1	1	1	1	1	1
ON06-255a	ON	1	1	1	1	1	1	1	1	1
ON06-255b	ON	1	1	1	1	1	1	1	1	1
ON06-260a	ON	1	1	1	1	1	1	1	1	1
ON06-262	ON	9	10	5	4	6	1	6	1	6
ON06-277	ON	9	10	5	4	6	1	6	1	6
ON06-292c	ON	**311**	8	1	1	7	1	1	1	10
ON06-293	ON	34	8	1	1	7	1	6	1	10
ON06-299	ON	3	4	1	1	1	1	6	1	7
ON06-30	ON	3	4	1	1	1	1	6	1	7
ON06-336b	ON	1	1	1	1	1	1	1	1	1
ON06-371b	ON	3	4	1	1	1	1	6	1	7
ON06-422	ON	29	18	12	1	11	2	15	1	2
ON06-66	ON	18	7	6	6	1	1	5	5	5

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TABLE 1—*Continued*

NML ID no.[a]	P/T[b]	ST[c]	Allele no.							
			clpA	*clpX*	*nifS*	*pepX*	*pyrG*	*recG*	*rplB*	*uvr*
ON06-67a	ON	57	25	4	3	16	3	21	1	2
ON06-90a	ON	50	8	1	1	17	1	6	1	10
ON07-1001	ON	1	1	1	1	1	1	1	1	1
ON07-1073	ON	8	5	5	4	5	5	5	1	6
ON07-1115	ON	9	10	5	4	6	1	6	1	6
ON07-1151	ON	3	4	1	1	1	1	6	1	7
ON07-1194	ON	1	1	1	1	1	1	1	1	1
ON07-1218	ON	1	1	1	1	1	1	1	1	1
ON07-1231	ON	37	7	6	12	1	1	5	5	5
ON07-124b	ON	1	1	1	1	1	1	1	1	1
ON07-128	ON	1	1	1	1	1	1	1	1	1
ON07-1289	ON	1	1	1	1	1	1	1	1	1
ON07-139a	ON	9	10	5	4	6	1	6	1	6
ON07-14	ON	**222**	5	5	4	5	1	15	1	6
ON07-147	ON	1	1	1	1	1	1	1	1	1
ON07-15	ON	**222**	5	5	4	5	1	15	1	6
ON07-150	ON	3	4	1	1	1	1	6	1	7
ON07-157b	ON	1	1	1	1	1	1	1	1	1
ON07-158a	ON	1	1	1	1	1	1	1	1	1
ON07-219	ON	**313**	10	5	4	116	1	15	1	6
ON07-223	ON	55	23	1	17	20	2	1	1	10
ON07-285	ON	1	1	1	1	1	1	1	1	1
ON07-289	ON	1	1	1	1	1	1	1	1	1
ON07-292a	ON	14	9	1	1	7	1	6	1	10
ON07-336	ON	14	9	1	1	7	1	6	1	10
ON07-351	ON	36	10	5	4	6	1	15	1	6
ON07-361	ON	4	8	1	1	1	4	6	1	7
ON07-399	ON	29	18	12	1	11	2	15	1	2
ON07-424	ON	1	1	1	1	1	1	1	1	1
ON07-430	ON	**307**	9	1	1	7	1	1	1	10
ON07-441	ON	**314**	14	1	11	1	113	1	1	10
ON07-471	ON	3	4	1	1	1	1	6	1	7
ON07-472	ON	3	4	1	1	1	1	6	1	7
ON07-507	ON	1	1	1	1	1	1	1	1	1
ON07-520	ON	19	4	4	3	3	3	3	3	3
ON07-576	ON	**222**	5	5	4	5	1	15	1	6
ON07-590	ON	9	10	5	4	6	1	6	1	6
ON07-600	ON	3	4	1	1	1	1	6	1	7
ON07-625a	ON	14	9	1	1	7	1	6	1	10
ON07-727	ON	14	9	1	1	7	1	6	1	10
ON07-766b	ON	1	1	1	1	1	1	1	1	1
ON07-791b	ON	8	5	5	4	5	5	5	1	6
ON07-795	ON	3	4	1	1	1	1	6	1	7
ON07-860	ON	3	4	1	1	1	1	6	1	7
ON07-861	ON	1	1	1	1	1	1	1	1	1
ON07-873	ON	**315**	8	1	1	17	1	6	95	10
ON07-88	ON	3	4	1	1	1	1	6	1	7
PEI05-15	PEI	**316**	10	1	4	6	1	6	1	6
PEI05-20	PEI	36	10	5	4	6	1	15	1	6
PEI05-30	PEI	9	10	5	4	6	1	6	1	6
PEI06-20	PEI	1	1	1	1	1	1	1	1	1
PEI06-23	PEI	8	5	5	4	5	5	5	1	6
PEI06-27	PEI	3	4	1	1	1	1	6	1	7
PEI06-34	PEI	18	7	6	6	1	1	5	5	5
PEI06-37	PEI	**221**	3	3	2	2	3	4	4	4
PEI06-48	PEI	19	4	4	3	3	3	3	3	3
PEI06-49	PEI	3	4	1	1	1	1	6	1	7
PEI06-55	PEI	59	6	1	5	1	1	7	1	19
PEI06-56	PEI	19	4	4	3	3	3	3	3	3
PEI06-67	PEI	3	4	1	1	1	1	6	1	7
PEI06-84	PEI	**317**	128	3	2	4	3	4	4	4
PEI06-85	PEI	1	1	1	1	1	1	1	1	1
PEI07-10	PEI	3	4	1	1	1	1	6	1	7
PEI07-24	PEI	7	6	1	5	1	1	7	1	8
PEI07-33b	PEI	3	4	1	1	1	1	6	1	7
PEI07-48	PEI	36	10	5	4	6	1	15	1	6

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TABLE 1—*Continued*

NML ID no.[a]	P/T[b]	ST[c]	Allele no.							
			clpA	*clpX*	*nifS*	*pepX*	*pyrG*	*recG*	*rplB*	*uvr*
PEI07-522	PEI	**318**	21	1	15	8	1	1	4	7
PEI07-74	PEI	19	4	4	3	3	3	3	3	3
PEI07-77	PEI	9	10	5	4	6	1	6	1	6
QC05-156	QC	1	1	1	1	1	1	1	1	1
QC07-344	QC	38	21	1	15	8	1	18	4	7
QC07-362	QC	34	8	1	1	7	1	6	1	10
QC07-399	QC	8	5	5	4	5	5	5	1	6
QC07-402	QC	**238**	10	5	75	6	1	15	1	6
QC07-484	QC	29	18	12	1	11	2	15	1	2
QC07-493	QC	3	4	1	1	1	1	6	1	7
QC07-565b	QC	4	8	1	1	1	4	6	1	7
QC07-595	QC	36	10	5	4	6	1	15	1	6
QC07-603	QC	8	5	5	4	5	5	5	1	6
QC07-650	QC	16	2	2	1	2	2	2	2	2
QC07-723	QC	11	5	7	5	1	6	1	4	9
QC07-755	QC	7	6	1	5	1	1	7	1	8
QC07-765	QC	3	4	1	1	1	1	6	1	7
QC07-776	QC	12	3	3	2	4	3	4	4	4
QC07-785	QC	1	1	1	1	1	1	1	1	1
QC07-815	QC	8	5	5	4	5	5	5	1	6
QC07-819	QC	8	5	5	4	5	5	5	1	6
QC07-83	QC	1	1	1	1	1	1	1	1	1
QC07-84	QC	3	4	1	1	1	1	6	1	7
QC07-851	QC	59	6	1	5	1	1	7	1	19
QC07-908	QC	1	1	1	1	1	1	1	1	1
QC07-951	QC	3	4	1	1	1	1	6	1	7
QC07-1008	QC	14	9	1	1	7	1	6	1	10
QC07-1048	QC	59	6	1	5	1	1	7	1	19
QC07-1054	QC	3	4	1	1	1	1	6	1	7
QC07-161-5	QC	1	1	1	1	1	1	1	1	1
QC07-175-3	QC	59	6	1	5	1	1	7	1	19
QC07-181-1	QC	14	9	1	1	7	1	6	1	10
QC07-181-4	QC	14	9	1	1	7	1	6	1	10
QC07-182-4	QC	14	9	1	1	7	1	6	1	10
QC07-182-6	QC	14	9	1	1	7	1	6	1	10

[a] All ticks were feeding adults except for those in italics, which were questing adults. NML, National Microbiology Laboratory.
[b] P/T, province or territory. AB, Alberta; MB, Manitoba; NB, New Brunswick; NS, Nova Scotia; NFL, Newfoundland and Labrador; PEI, Prince Edward Island; ON, Ontario; QC, Quebec.
[c] STs in boldface and underlined are new to this study, although data from all but one of the ticks collected in Quebec have been presented previously (31). The STs in boldface were present in the MLST database but had not been further analyzed.

(Table 1) (15). Two STs dominated the sample set (Table 2): ST1 ($n = 35$) and ST3 ($n = 26$). The next highest frequencies were found for ST14 ($n = 11$) and ST9 ($n = 11$), and all of these STs had been found in the NE United States previously.

Samples carrying STs that had been described only from NE regions of the United States were not found west of 80°W. Samples carrying STs that had been described only from MW regions of the United States were not found east of this longitude, except in the case of two ticks that came from Quebec. Ticks carrying MW STs formed a significant cluster (relative risk [RR] = 56.67; $P = 0.001$) centered on 49.77°N and 97.32°W, with a radius of 1,511 km and encompassing ticks collected in Alberta, Manitoba, and western Ontario to Simcoe (Fig. 1).

In total, 19 new STs were identified, 9 of which showed new allelic profiles, while the other 10 contained one or more new alleles (Table 1). Three STs were present in the MLST database but have not yet been further described (i.e., ST221, ST222, and ST226), so we consider them new to the present study. The ratio of new STs to previously described STs was 3/6 (50%) in Manitoba, 8/67 (12%) in Ontario, 1/31 (3.2%) in Quebec, and 8/61 (13%) in the Atlantic region. ST222 was found in Ontario east and west of 80°W and ST221 in Manitoba and on Prince Edward Island (Table 1), and ST301 has been found in questing ticks sampled in Wisconsin (unpublished data). A significant cluster of new STs (RR = 5.15; $P = 0.005$) was identified centered on 53.54°N and 113.49°W, which encompassed ticks collected in Alberta, Manitoba, and Ontario, except for ticks from southeastern Ontario along the St. Lawrence valley (Fig. 1). Within this cluster, 12/27 (44.4%) ticks carried new STs, while outside the cluster, 12/139 (8.6%) ticks carried a new ST. In logistic regression analysis, occurrence within the cluster explained the occurrence of new STs better than the simpler explanatory variable of longitude ($\chi^2 = 5.2$; degree of freedom [df] = 1; $P < 0.01$).

Several clonal complexes (CC) were identified in goeBurst (Fig. 2); some had been described previously by Hoen and colleagues (15) (i.e., CC34, CC37, and CC19), but there were also new ones (CC36 and CC12) through connection of new STs with potential founder strains originating in the NE United States (Fig. 2). However, the bootstrap values for founder assignments were 64% or less (data not shown), which

TABLE 2. Frequencies of *B. burgdorferi* STs in the sample of ticks collected in surveillance in Canada

ST	Frequency	Geographic region[a]	New ST	Geographic region of nearest ancestor for new STs
1	35	NE	No	
3	26	NE	No	
4	4	NE	No	
7	6	NE	No	
8	7	NE	No	
9	11	NE	No	
11	1	NE	No	
12	3	NE + MW	No	
14	11	NE	No	
16	2	NE	No	
18	5	NE	No	
19	6	NE	No	
29	4	NE + MW	No	
34	2	NE	No	
36	7	NE	No	
37	2	NE	No	
38	3	NE	No	
50	1	NE	No	
55	1	MW	No	
57	1	NE	No	
59	5	NE	No	
221	2	NE + MW		NE + MW
222	3	NE + MW		NE
226	1	NE		MW
238	1	NE	Yes	NE
300	1	MW	Yes	MW
301	1	MW	Yes	MW
302	1	MW	Yes	NE
305	1	NE	Yes	NE
306	1	NE	Yes	NE
307	2	NE	Yes	NE
308	1	NE	Yes	NE
309	1	NE	Yes	NE
311	1	NE	Yes	NE
313	1	NE	Yes	NE
314	1	NE	Yes	MW
315	1	NE	Yes	NE
316	1	NE	Yes	NE
317	1	NE	Yes	NE + MW
318	1	NE	Yes	NE

[a] NE, STs already identified in NE United States; MW, STs already identified in MW United States; New ST, STs identified for the first time in this study. Also shown for STs new to this study is the geographic region of occurrence of the nearest ancestor.

likely reflects the low number of SLV descending from each founder. This is consistent with the goeBurst data, in which higher levels of tie break rule (indicated by different colors in Fig. 2) refer to lower levels of confidence in the link.

Nevertheless, most of the new STs identified in the data set analyzed here were single-locus (ST300 to ST302, ST305 to ST308, ST311, ST313, and ST315 to ST318), double-locus (ST309, ST226, and ST222), or triple-locus (ST314) variants of previously described STs (Fig. 3). Most strains linked in the same CC also clustered in the same clade in the phylogenetic tree. The exceptions were ST52, ST53, and ST301, which were assigned to CC34 by goeBurst but fell into a different clade in the phylogenetic tree (Fig. 3). ST281 was genetically divergent from *B. burgdorferi*, showing new alleles for all eight housekeeping genes (Table 1), and phylogenetic analysis revealed that this ST is more closely related to *Borrelia bissettii* than to *B. burgdorferi* and belongs, in fact, to a new species within the *B. burgdorferi* species complex (Fig. 3) (26).

***ospC* analysis.** Of the 400 tick samples available to the study, *ospC* alleles were obtained from 309. All 17 known alleles were represented in the sample, but alleles A, K, and N, which have all been associated with disseminated Lyme disease (33), were dominant (Fig. 4). A spatial cluster of ticks carrying allele I was observed in Nova Scotia centered on 45.09°N and 64.37°W, with a 67.51-km radius (Fig. 5). A spatial cluster of ticks carrying allele L was observed that extended from Manitoba into western Ontario, centered on 48.72°N and 94.57°W, with a 1,282-km radius (Fig. 5). There was no significant spatial clustering of ticks carrying other alleles.

More than one *ospC* allele was identified in 108 ticks (60 with 2 alleles, 36 with 3 alleles, 10 with 4 alleles, 1 with 5 alleles, 1 with 6 allele, and 2 with 7 alleles). In pairwise analyses, ticks infected with *B. burgdorferi* carrying allele F were significantly more likely to be coinfected with *B. burgdorferi* carrying allele M (odds ratio [OR] = 2.99; 95% confidence interval [95% CI], 0.1 to 0.77; $P = 0.047$), and coinfections in the same tick by *B. burgdorferi* carrying alleles G and H, J and T, and L and O were more likely to occur than by chance (OR = 3.29, 10.60, and 9.09; 95% CI, 1.3 to 8.1, 1.0 to 107, and 1.7 to 498; $P = 0.010$, 0.046, and 0.011, respectively). Coinfections by *B. burgdorferi* carrying alleles A and F were significantly less likely to occur in the same tick (OR = 0.26; 95% CI, 0.1 to 0.8; $P = 0.015$). No significant positive or negative three-way (or more complex) relationships were found. These findings are summarized in Table 3. Correspondence analysis supported the significance of these pairwise comparisons when the full variance-covariance matrix of the data was accounted for: all positively associated pairs occurred in the same parameter space, while the negatively associated pair (A and F) occupied very separated parameter spaces (Fig. 6).

DISCUSSION

In this study, we identified a wide diversity of *B. burgdorferi* STs, as well as the presence of other tick-borne *Borrelia* spp. These findings have significance for the diagnosis of Lyme disease, and the possible occurrence of disease caused by other tick-borne bacteria, in eastern and central Canada.

First, this study determined that, as well as carrying *B. burgdorferi*, *I. scapularis* ticks collected in Canada also carry relapsing-fever-like spirochetes, most likely *B. miyamotoi*. The implications for public health are currently uncertain, because it is not known whether this species is pathogenic to humans. The prevalence of ticks infected with these spirochetes in nature is likely to be higher than revealed in our study: the screening PCRs would not have been positive if ticks had been infected only with *B. miyamotoi*. We identified only ticks coinfected with *B. miyamotoi* and *B. burgdorferi*, not ticks that were infected with *B. miyamotoi* alone. Studies on questing *I. scapularis* in the United States have shown an approximate 1:10 ratio of *B. miyamotoi* to *B. burgdorferi* (3), while in our study the ratio was 1:24.

Second, one tick carried an entirely new *Borrelia* sp. (ST281), which was most closely related to *B. bissetii*, and we have proposed that it be named *Borrelia kurtenbachii* (26). Strains belonging to this bacterial species found in the United

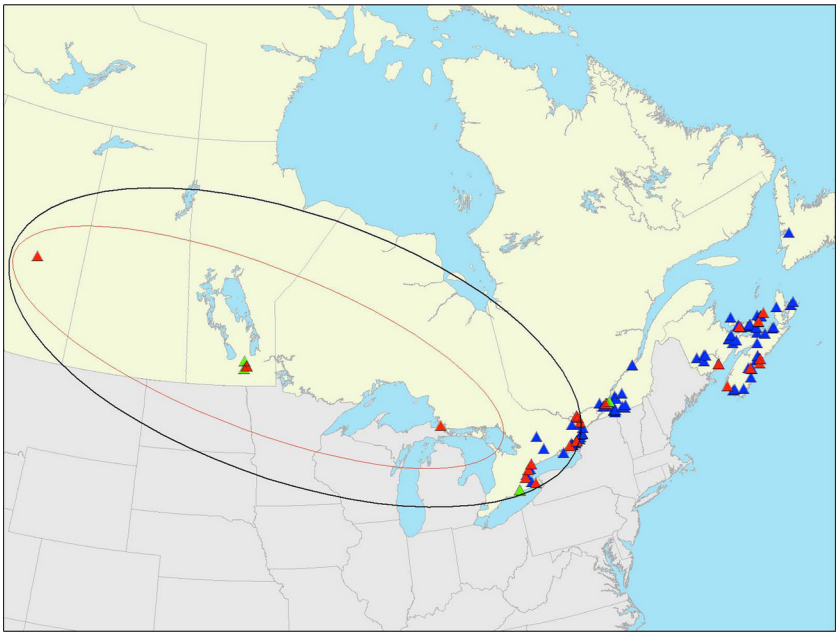

FIG. 1. Geographic distribution of sites of collection of ticks carrying the different MLST STs of *B. burgdorferi* identified in the study. The triangles indicate the locations of ticks carrying those STs already identified in the NE United States (blue triangles) and those STs already identified in the MW United States (green triangles) and ticks carrying STs identified for the first time in this study (red triangles). The black ellipse indicates the geographic locations of ticks that formed a spatial cluster that carried new STs, and the red ellipse indicates the geographic locations of ticks that formed a spatial cluster that carried STs already identified in the MW United States.

States, e.g., 25015, have been shown to be mildly pathogenic in mice (1, 10). However, determining that ticks collected in Canada can carry this *Borrelia* sp. and that ticks in Canada can carry *B. miyamotoi* raises the possibility (which needs further study) that infections in humans for which we currently have no diagnostic methods could be occurring in Canada. Therefore, the possible presence of these species needs to be taken into consideration in diagnosis of human and animal patients.

Third, there was a high degree of diversity of *B. burgdorferi*, with 40 STs identified, 16 of which were entirely new while 3 were present in the MLST database but have not been further analyzed. Variations in clinical symptoms of affected humans, as well as differences in performance of serodiagnostic tests, are seen among European species of the *B. burgdorferi* sensu lato complex (34). These species frequently have different natural reservoir hosts, mostly due to their capacity to resist the alternative pathways of complement of different host species (21, 22, 23). It is unknown what confers the ability among Lyme disease spirochetes to cause disease in humans and to what extent ecological differences directly drive clinical differences. Although precise determinants of pathogenicity are unknown, differences in pathogenicity among different *B. burgdorferi* genotypes from the United States have been demonstrated (18, 33, 36, 37, 38), as well as the fact that the performance of some serodiagnostic tests varies among genetic variants (17, 39). While *B. burgdorferi* remains a generalist in North America (13), some studies suggest evidence of early signs of multiple-niche polymorphism (4), and we have identified a number of possible drivers for adaptive radiation of *B. burgdorferi* in North America (23). These include climate effects on tick seasonality (28), for which there may be evidence from the geographic variation in the occurrence of ribosomal sequence types (RSTs) in the United States (15).

Studies on differential pathogenicity (i.e., the ability or lack of ability to cause disseminated Lyme disease) of *B. burgdorferi* genotypes or different abilities of infected individuals to produce antibodies that are detectable in standard serological assays, have mainly focused on differences among RSTs (14, 38). RSTs can be directly assayed from IGS types or inferred from *ospC* alleles because of linkage disequilibrium among these loci, which has been observed (at least in the northeastern United States [6, 14]). The wide range of *ospC* alleles found in our study suggests that variations in pathogenicity and sensitivity to serological tests can be expected in Canadian patients, since it is also observed in the United States (8, 37, 38, 39). To date, MLST typing has not been used to identify genotypes of *B. burgdorferi* that differ in the ability to cause disseminated disease in humans or to elicit immune responses detectable by commercial test kits. However, the utility of MLST analysis in this regard may be worth investigating, as studies in Europe suggest that MLST is able to detect phenotypic differences among *B. burgdorferi* sensu lato spirochetes in terms of identifying ecotypes that vary in the ability to infect different reservoir hosts (25).

For those STs common to the United States and Canada, the geographic (at least longitudinal) patterns of their occurrence were similar in the two countries. While a number of new STs were identified, the goeBurst analysis clustered many of the new STs as SLV to the previously described CC34, −37, −19, and −7 with founders in the United States (15), suggesting a close relationship of the populations. Two new complexes, CC36 and CC12, were formed due to STs being SLV or DLV

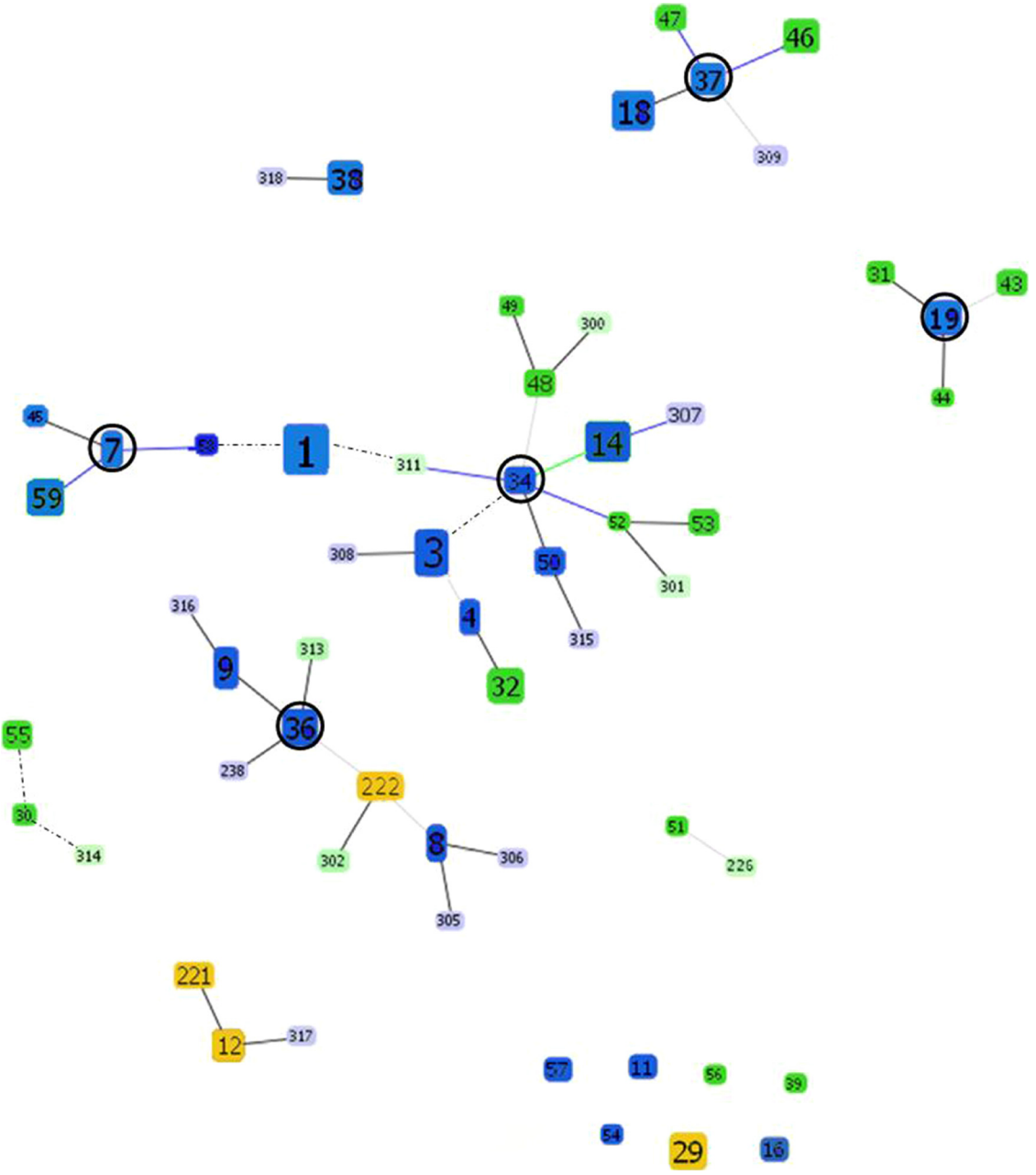

FIG. 2. Population snapshot of 244 samples of *B. burgdorferi* found in Canada (166 samples) and the Unites States (78 samples) as determined by goeBurst analysis. Each colored dot corresponds to an ST: the dark-green dots are STs previously found in the upper MW United States, the dark-blue dots are STs previously found in the NE United States, the light-green STs are found in Canada west of 79°W, and the light-blue STs are found in Canada east of 79°W, while the orange dots indicate STs found in both the eastern and middle western regions of Canada or the United States. The size of the dot corresponds approximately to the sample size. The lines link related STs (clonal complexes). Potential founders of clonal complexes are circled in black. Colored lines connecting STs indicate descending order of certainty: black lines are inferred without tie break rules, blue lines are inferred using tie break rule 1 (number of SLV), and green lines are inferred using tie break rule 2 (number of DLV). The light-gray lines correspond to DLV. TLV are indicated by dashed lines. The majority of the new STs found in the Canadian sample set are linked to previously defined STs.

of North American ST36, -8, -9, and -12. For individual complexes, the founder assignment and direction of descent could not be established with certainty due to a paucity of SLV associated with complex founders. This does not invalidate the CCs, it simply means that the direction of descent, i.e., founder/subfounder assignment, may change when more SLV are added. Further sampling and additions to the *B. burgdorferi* MLST database are likely to lead to denser "forests" and better resolution of clonal complexes, which would improve the inference of founders and descendants. Such enhanced data sets will likely aid resolution of the phylogeography of *B. burgdorferi* in North America, which appears complex. This

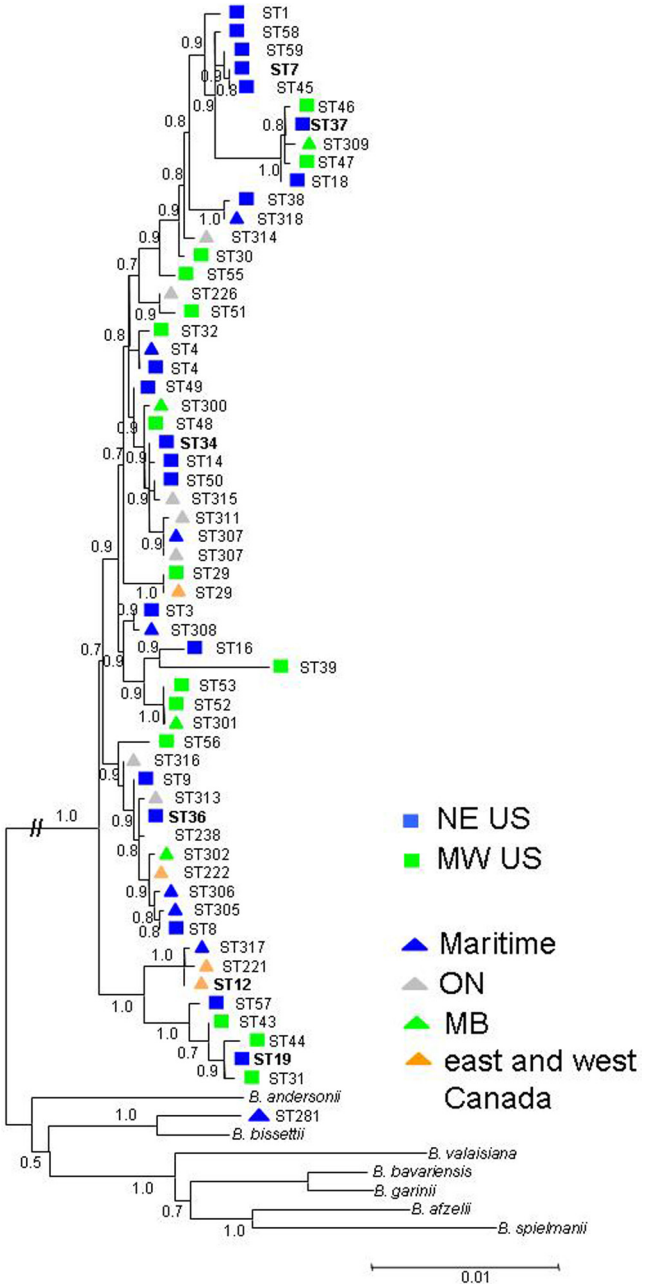

FIG. 3. ST tree of *Borrelia* samples collected in the United States and Canada. The tree was constructed using concatenated sequences of eight housekeeping genes for maximum-likelihood analysis (PhyML). The outgroup branch is not according to scale, as indicated by slashes. The scale bar indicates 1% divergence.

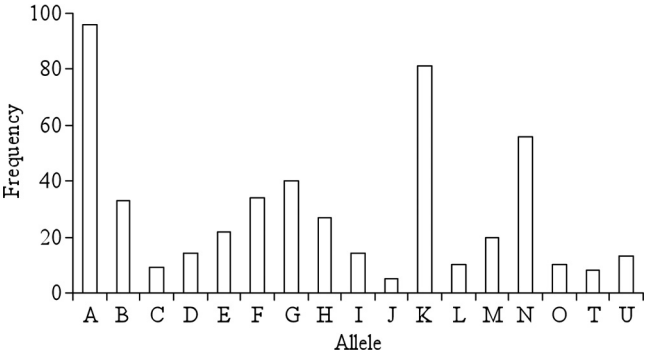

FIG. 4. Frequency distribution of *ospC* alleles identified in ticks in this study.

complexity may be due to repeated expansions and contractions of the geographic range of *B. burgdorferi* (perhaps associated with glacial-interglacial cycles), which result in observations on long- and short-term evolution of *B. burgdorferi* in North America that at first sight appear to conflict (5, 15). Nevertheless, in our study, the likely ancestor identified in all the clonal complexes was an ST found in the United States. For the most part, the complexes followed the geographic pattern expected from (15), i.e., NE STs being ancestors of MW STs and STs new to this study having ancestors that originated from the same longitude in the United States (Fig. 3). Therefore, the goeBurst analysis was consistent with the general pattern of south-north transportation of *B. burgdorferi* in ticks suggested by cluster analysis. Earlier studies of *B. burgdorferi* diversity, which analyzed outer surface protein sequence variations, identified latitudinal conformity of *B. burgdorferi* in the eastern United States (32). This was interpreted as balancing selection predominating over migration as a driver of diversity. Our study, using MLST of housekeeping genes with nearly neutral variation and employing ticks collected across a wide longitudinal range, suggests that south-north migration (in the case of Canada) may indeed be an explanation for latitudinal conformity.

In a previous study by Hoen et al. (15) that had similar sample sizes from the NE ($n = 41$) and MW ($n = 37$) United States, 37 unique STs were identified, 20 of which were found in the NE and 17 in the MW. The finding of more new STs in western parts of Canada in the present data set may be related to differences in sampling efforts in the United States and Canada. In the United States, field-collected ticks (mostly nymphs) were analyzed, while in Canada, the samples were mostly adult ticks obtained by passive surveillance. Many of these ticks, and the *B. burgdorferi* isolates they carry, were likely carried into Canada from the United States by migratory birds (29). However, some ticks are likely to have come from endemic populations in Canada. Therefore, an alternative explanation would be that some *Borrelia* populations in Canada, for example, at Long Point, Lake Erie (30), are refugial populations that have been isolated for long enough to accumulate mutations and are now themselves spreading their geographic ranges. The fact that a circular cluster, rather than simply accounting for a longitudinal cline, explained the spatial distribution of new STs in Canada may support this hypothesis.

Geographic variation in the occurrence of *ospC* alleles was in part similar to that seen in the United States. For example ticks carrying allele L occurred mostly in the western range of *I. scapularis* collected in passive surveillance, while allele I occurred only in eastern Canada, similar to findings in the United States (5). Nevertheless, spatial clustering of ticks carrying *ospC* alleles I and L (which was particularly spatially constrained for allele I) could indicate spatial variation in *ospC*

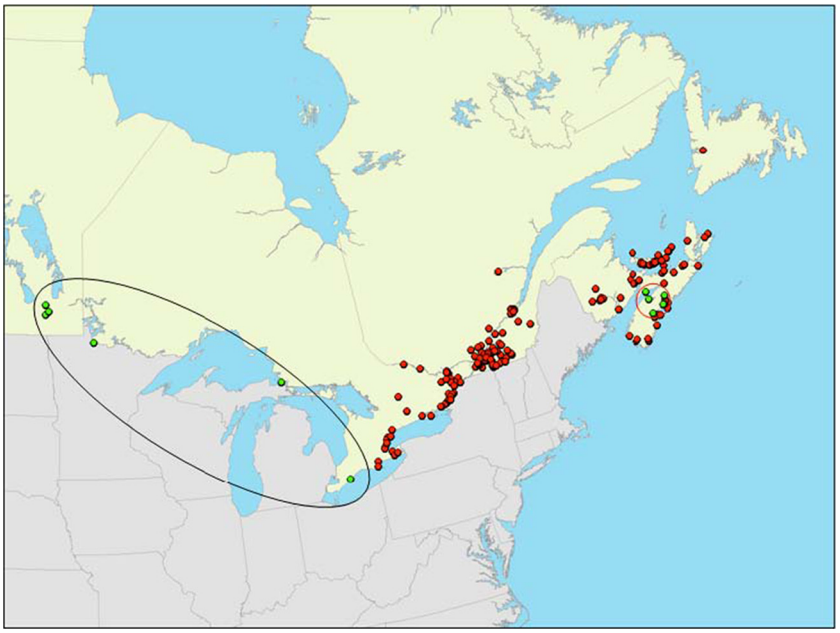

FIG. 5. Geographic distribution of sites of collection of ticks carrying different *ospC* alleles identified in the study. A significant spatial cluster of ticks infected with *B. burgdorferi* carrying allele L is indicated by the black ellipse, and a significant spatial cluster of ticks infected with *B. burgdorferi* carrying allele I is indicated by the red circle.

allele frequencies associated with founder events that are likely to be occurring at present in Canada (31).

The spatial pattern of some *ospC* alleles and the occurrence of positive and negative associations between some alleles in the same tick may support suggestions of multiple-niche polymorphism. Coinfections of adult ticks with two alleles occurred more frequently than by chance in some cases. This could suggest that immature ticks fed on hosts that were coinfected with *B. burgdorferi* carrying *ospC* alleles that were associated with specificity for similar host species. We speculate that variations among species in mounting an effective immune response to different *ospC* alleles could be one mechanism for host specialization (17). Negative associations among *ospC* allele coinfections could also indicate differing host specificities of *ospC* alleles by indicating particularly cross-protecting immune responses among some *ospC* alleles.

Our study shows that there is a wide range of *Borrelia* species in ticks collected in Canada, and until proved otherwise, they must be considered a potential risk to public health. The study was based on ticks collected in passive surveillance, and the proportion of ticks that originated from locations in Canada where ticks are endemic versus those that may have been dispersed from the United States by migratory birds is mostly unknown. Assuming that the ticks came from both sources, our study shows that *B. burgdorferi* isolates in ticks collected in Canada have a wide genetic diversity. The diversity observed mostly reflected that found in the United States (in terms of

TABLE 3. Numbers of ticks infected with *B. burgdorferi* that carried pairs of *ospC* alleles for each possible pairing

Allele	Total no. of infected ticks carrying allele	No. of ticks with pairing[a]															
		B	C	D	E	F	G	H	I	J	K	L	M	N	O	T	U
A	96	8	2	4	5	**4**$^{-}$	11	5	3	0	21	3	5	16	2	4	4
B	33		1	0	1	4	2	2	2	1	11	0	0	3	0	2	0
C	9			1	1	1	1	1	0	0	0	0	0	1	1	1	1
D	14				1	0	2	2	0	1	2	1	0	2	1	0	1
E	22					2	3	1	2	1	4	1	2	2	2	1	1
F	34						3	2	3	0	8	1	**5**$^{+}$	2	1	0	0
G	40							**8**$^{+}$	4	0	12	1	4	4	2	2	3
H	27								3	0	4	0	2	2	0	0	0
I	14									0	4	0	1	0	0	1	1
J	5										0	0	0	1	0	**1**$^{+}$	0
K	81											0	8	14	3	2	0
L	10												0	1	**2**$^{+}$	0	0
M	20													5	1	0	0
N	56														1	2	1
O	10															0	0
T	8																0
U	13																

[a] Significant associations are in boldface; positive associations are indicated by a superscript + and negative associations by a superscript minus sign.

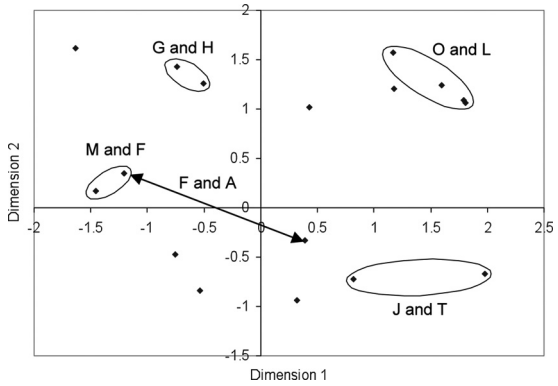

FIG. 6. Graphical representation of the results of correspondence analysis of *ospC* allele pairwise comparisons showing that positively associated pairs (indicated by ellipses) occur in the same parameter space while a negatively associated pair (indicated by the double-headed arrow) occurs in different parameter spaces.

degree and geographic location), where multiple long- and short-term evolutionary processes are in play. Our study does, however, suggest the possibility that in Canada long-term evolutionary processes (e.g., the possible occurrence of refugial populations following glacial contractions of the *B. burgdorferi* geographic range) and short-term evolutionary processes (e.g., founder events) shape a population structure of *B. burgdorferi* that is distinct from that in the United States. This means that the genetic diversity of *B. burgdorferi* in eastern and central Canada could be clinically or diagnostically significant. More prospective field studies in existing and emerging areas where Lyme disease is endemic, combined with studies on the diversity of *B. burgdorferi* in Canadian Lyme disease patients, are needed to elucidate the processes of evolution of *B. burgdorferi* in Canada and their significance for public health.

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